Enniatin Production by *Fusarium tricinctum* and its Effect on Germinating Wheat Seeds

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ABSTRACT

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Fusarium tricinctum strains isolated from winter wheat with symptoms of crown and root rot, English ivy with leaf spot, apparently healthy red clover root, or pasture soil produced enniatins. Although enniatins are metabolites of at least five other species in the genus, this is the first report of their production by *F. tricinctum*. Analysis by mass spectrometry/mass spectrometry of extracts from 13 isolates showed that 10 of them were positive for enniatin with concentrations ranging from about 3 to 3,270 μg/g of corn substrate. When enniatin (10–80 μg/ml) was added to the solution in which wheat seeds were germinated, growth reduction of the developing seeds was directly related to the concentration of enniatin, with root elongation being inhibited more than leaf development.

Additional key word: phytotoxin.

An investigation of antibiotic-producing fungi by Gäumann et al (9) led to the discovery of metabolites that they named enniatins, after the producing fungus, *Fusarium orthoceras* Appl. & Wr. var. *enniatinum*, later renamed *F. oxysporum* Schlect. (17). Enniatins, hexadepsipeptides with alternating residues of 2-hydroxy-isovaleric acid and branched *N*-methyl amino acids (Fig. 1), have antibiotic (9,20), biological membrane-modifying (13,18), insecticidal (10), and phytotoxic (8) properties. One or more of these metabolites are produced by species distributed in five of the 12 sections of the genus *Fusarium* sensu Marasas, Nelson, and Toussoun (14). In addition to *F. oxysporum* section (Elegans), the following species within various sections of the genus were found to elaborate enniatins: *F. acuminatum* Ell. & Ev. (5) (Gibbosum), *F. sambucinum* Fuckel (1,2,7,15) (Discolor), *F. avenaceum* (Fries) Sacc. (7,20) (Roseum), and *F. lateritium* Nees (3,8) (Lateritium). We have now added the species *F. tricinctum* (Corda) Sacc. (Sporotrichiella). To the best of our knowledge, none of the four species, *F. chlamydosporum* Wr. & Rg., *F. poae* (Peck) Wr., *F. sporotrichioides* Sherb., and *F. tricinctum*, comprising the section Sporotrichiella has previously demonstrated enniatin biosynthesis.

The predominant interest in the genus stems from the involvement of many species in plant diseases. *Fusarium* species cause root rots, wilts, and leaf spots and are serious pathogens on Gramineae, causing postemergence blighting (4,6,12,21). On wheat, several *Fusarium* species, frequently more than one in the same growing season, parasitize an individual plant. *F. tricinctum* is a secondary pathogen of wheat, and it may contribute to symptoms known as root and crown rot (12). Several of the species that parasitize wheat elaborate enniatins, nonhost specific phytotoxins. Their role in plant diseases has not been established (5,19), although much has been learned about their role as membrane-active compounds and as metal ion chelators. They apparently have not been studied since 1960, when Gäumann et al (8) demonstrated that mixtures of enniatins acted synergistically in causing wilt symptoms of excised tomato shoots. Because enniatins caused wilt symptoms and proved in our study to be common metabolites of *F. tricinctum* strains, we decided to determine if enniatin affected the development of germinating wheat seeds.

MATERIALS AND METHODS

Sources of strains and enniatin production. *F. tricinctum* strains NRRL 26430, 26562, 26563, 26564, and 26565 were obtained from Kane (12). These strains parasitized winter wheat grown in upper New York state and were identified by Kane according to the criteria of Marasas et al (14). Strains NRRL 26506 (ATCC 38179), NRRL 26507 (ATCC 38180), NRRL 26508 (ATCC 38181), NRRL 26509 (ATCC 38182), and NRRL 26510 (ATCC 38183) are ATCC cultures (11) isolated by El-Gholl et al (6) from leaf spot of English ivy growing in Florida. Strains NRRL 13434 (T-387), NRRL 13435 (T-388), and NRRL 13442 (T-546) are from the *Fusarium* Research Center Collection, The Pennsylvania State University (16). T-388 was isolated from barley in Finland (16), T-387 from red clover in Germany, and T-546 from pasture soil in Australia (P. Nelson, Pennsylvania State University, *personal communication*). Strains are maintained by the Agricultural Research Service Culture Collection (NRRL), Peoria, IL, as well as the culture collections from which they were obtained.

A white corn grit (WCG) medium was prepared in a 300-ml Erlenmeyer flask by adding 15 ml of water to 50 g of WCG before autoclaving and 10 ml of sterile water after the medium cooled. Each *F. tricinctum* strain was transferred to a V-8 agar slant and incubated at ambient light and temperature (17–23 C) for 10–12 days before inoculating the medium with 0.5 ml of the culture surface slurried in 5 ml of water. The inoculated WCG was incubated for 16 days at ambient light and temperature. For mass spectrometry/mass spectrometry (MS/MS) and thin-layer chromatographic (TLC) analyses, the contents of duplicate flasks of each strain were transferred with a liter of methanol to a Waring blender jar and macerated. The ground culture material was filtered and thoroughly rinsed with methanol. The volume of filtrate (860–950 ml) for each strain was measured, and aliquots (1 ml for MS/MS and 10 ml for TLC) were dried and redissolved in methanol for analysis.

Recovery and purification. For production of recoverable quantities of pure enniatin, strain NRRL 26430 was grown on WCG for 16 days at 20–22 C, extracted with methanol, filtered,

and concentrated. The residue was dissolved in chloroform and washed several times with water. The chloroform was evaporated, and the residue washed with hexane and then with acetonitrile. After rinsing in acetonitrile, the solids were dissolved in boiling acetonitrile and filtered, and the filtrate was placed in a −18 C freezer. Within 16 hr, crystals formed. Crystals were thoroughly rinsed with hexane and acetonitrile. Crystalline enniatin (crystals were verified as enniatin by MS, MS/MS, and nuclear magnetic resonance [NMR] [Fig. 2A and B]) used for TLC standards was recrystallized from acetonitrile. The criterion of purity was a single spot when chromatograms were subjected to iodine vapor, and no additional spots after spraying with 50% sulfuric acid in methanol and heating to 120 C for 15 min. Chromatograms were developed on 0.25-mm thick silica gel-60 precoated plates without fluorescent indicator (E. Merck, Darmstadt, West Germany) in either chloroform-methanol (4:1) or in ethyl acetate-methanol-water (8.5:1.0:0.5). The smallest detectable quantity of enniatin was determined by applying 1,000, 500, 250, and 125 ng of enniatin to a TLC plate, developing 10 cm in chloroform-methanol, and placing the sample in a tank of iodine vapor for 24 hr.

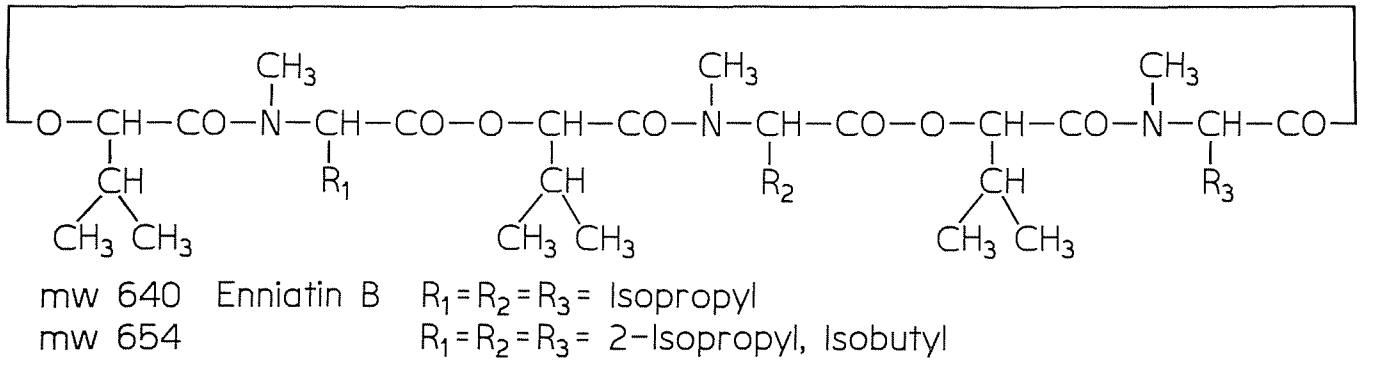

Fig. 1. Generalized structure of enniatins.

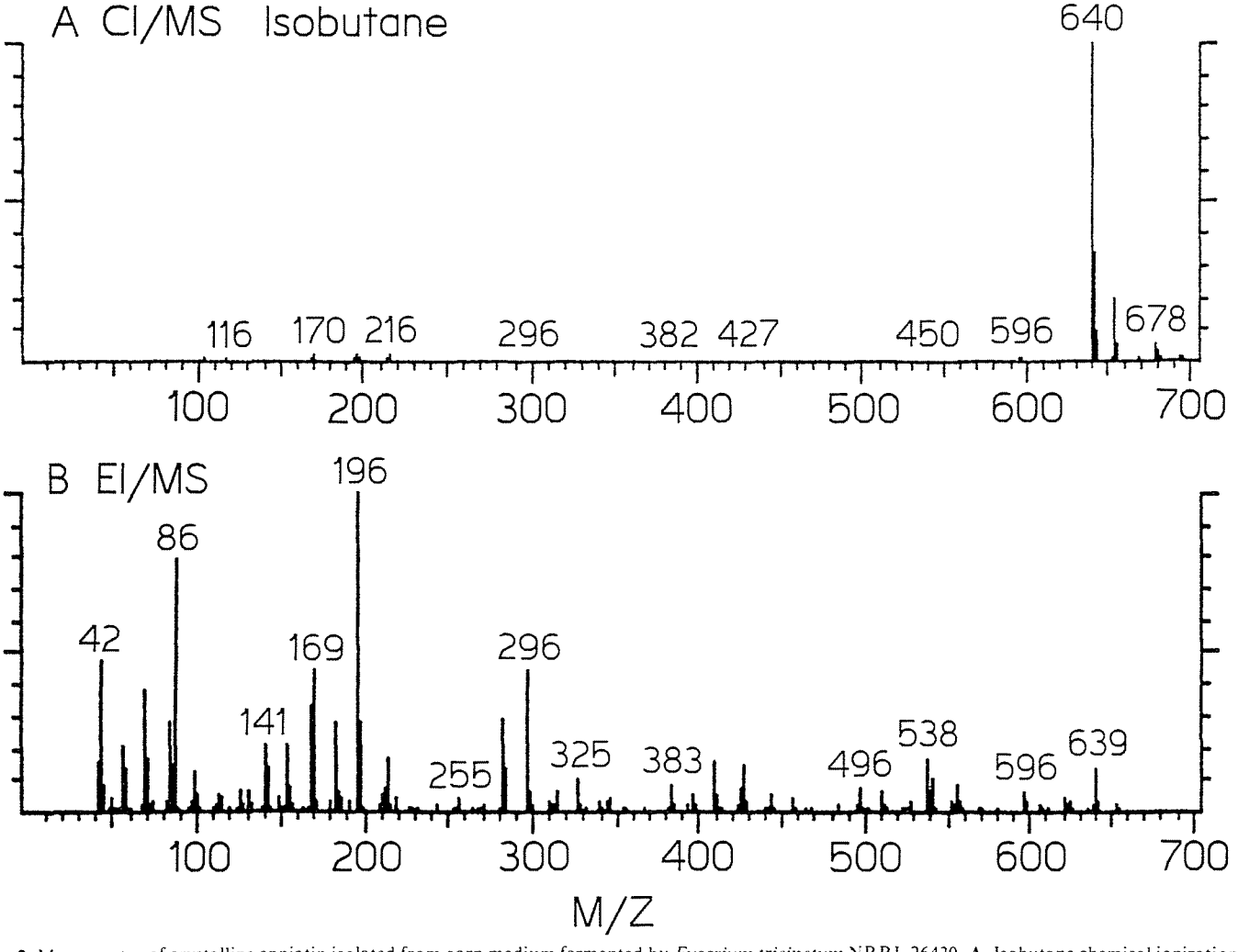

Fig. 2. Mass spectra of crystalline enniatin isolated from corn medium fermented by *Fusarium tricinctum* NRRL 26430. **A,** Isobutane chemical ionization spectrum and **B,** 70 V electron impact spectrum.

Mass spectrometry and nuclear magnetic resonance spectrometric analyses. One milligram of crystalline enniatin was dissolved in 0.25 ml of 6 N HCl and hydrolyzed by heating at 110 C for 16 hr in a sealed ampule. TMS derivatives of the hydrolysis products were analyzed directly by GC/MS. Mass spectra were recorded with a Finnigan TSQ-46 mass spectrometer. The direct exposure probe was used to admit samples into the mass spectrometer. Isobutane was the reagent gas for positive and negative CI spectra. The source temperature (as read by the instrument's source thermocouple) was 1,000 C, and the source pressure was 0.3 torr. For MS/MS experiments, argon was used as the collision gas. The pressure in the reaction cell was 2 mtorr and the collision energy was 20 V. NMR spectra were recorded in $CDCl_3$ on a Bruker WM300 instrument (Bruker Instruments Inc., Billerica, MA).

The levels of enniatins in crude extracts were estimated by comparison of the abundance of the most intense daughter signal (m/z 196) of the protonated enniatin B molecule (m/z 640) in the crude extract with the response for pure standard. An aliquot of extract equivalent to 1 mg of the original culture was used for the MS/MS experiment. The detection limit for pure enniatin was approximately 1 ng. This rapid protocol afforded a detection limit of about 1 ng/g of substrate with no sample cleanup of the methanol extract.

Growth inhibition assessment. Wheat kernels (*Triticum aestivum* L.) cultivar Arthur were surface sterilized by submerging them in 0.26% sodium hypochlorite for 5 min and rinsing them three times in sterile water. Fifty seeds per treatment, 10 per 9-cm petri dish, were placed on a Whatman No. 1 filter paper (Balston Filter Products, Lexington, MA). Enniatin was dissolved in dimethyl sulfoxide (DMSO), and 0.25 ml was added to 24.75 ml of water (1% DMSO) and diluted to give concentrations of 5, 10, 20, 40, and 80 μg/ml. Five milliliters of the proper dilution was added to each petri dish and the kernels covered with a Whatman No. 1 filter paper. Seeds were incubated in the light at room temperature. After a 6-day incubation, the seminal root and the primary leaf were measured.

RESULTS

Identification of product. Crystalline material isolated from fermentations of *F. tricinctum* NRRL 26430 was identified as enniatin B with the possible presence of trace amounts of one or more analogs of enniatin B. The isobutane chemical ionization spectrum of the material (Fig. 2A) had abundant signals only at m/z 640 (100) and m/z 654 indicating protonated molecules. The electron impact spectrum (Fig. 2B) had abundant molecular ion (m/z 639 [15]) and fragments across the mass range. Computerized library search results and the masses of observed fragments suggested that the material might be a polypeptide of about three to seven amino acids. A search in Chemical Abstracts among possible molecular formulas for compounds with a molecular weight of 639 with three to seven nitrogens revealed references to a family of cyclohexadepsipeptides (Fig. 1) called enniatins (9). The MS/MS collisionally activated daughter spectrum of m/z 640 (Fig. 3) had abundant daughters at m/z 527 (25, parent-113), 427 (20P-213), 214(60), and 196(100). These daughters are consistent with expected cleavages at the amide and ester bonds in an enniatin cyclohexadepsipeptide. This structure assignment was further supported by the negative CI mass spectrum, which showed the molecular anion at m/z 639(100) and sequence fragments at m/z 539(30, M-100), 426(27, M-213), and 212. Hydrolysis of the crystalline material yielded hydroxyisovaleric acid and *N*-methyl valine, the subunits of enniatin B.

Both the proton and carbon 13 NMR spectra were consistent with an enniatin structure. Because the three repeating units in the molecule are not completely equivalent environments, the signals were somewhat broadened, and only a third as many proton and

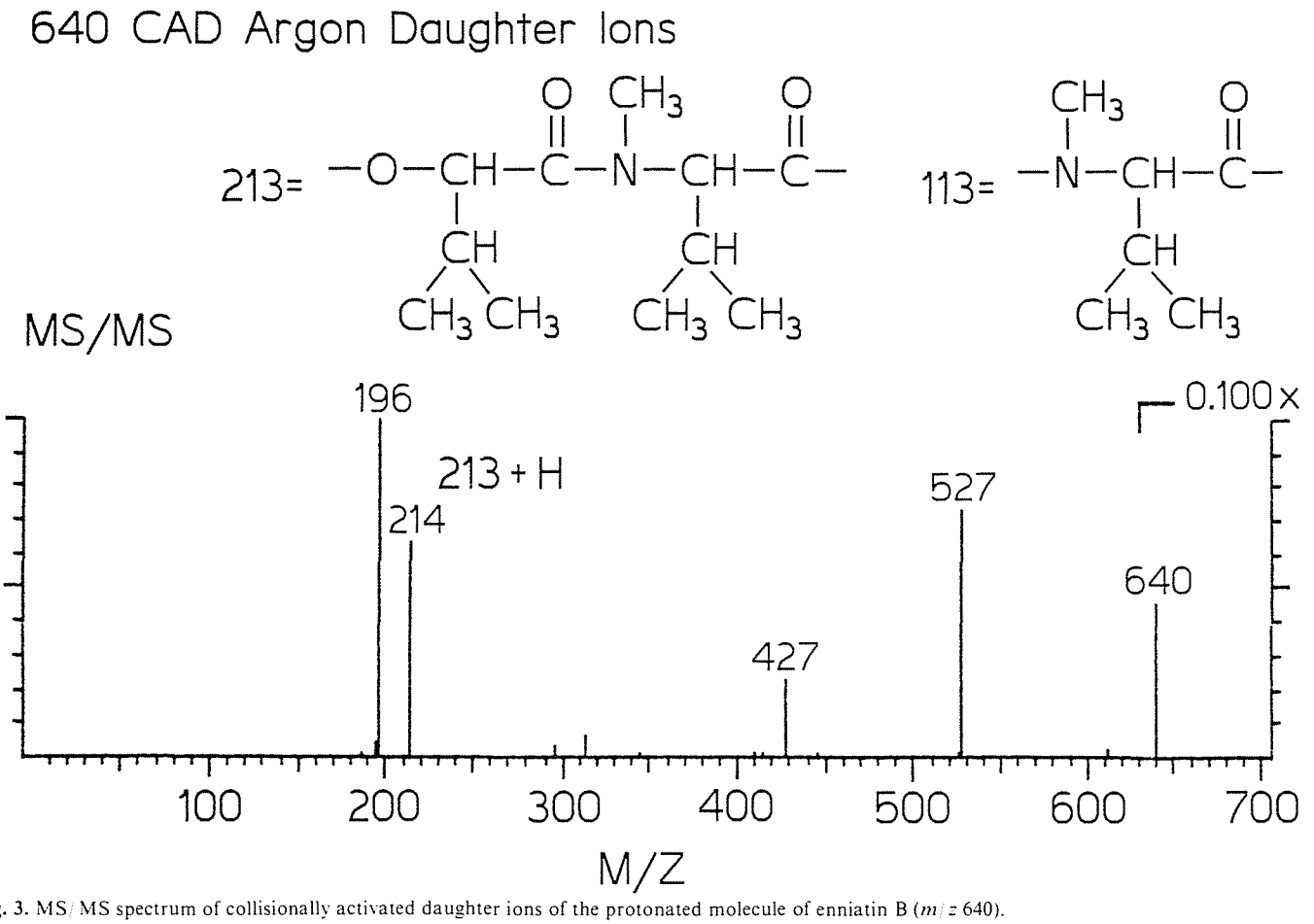

Fig. 3. MS/MS spectrum of collisionally activated daughter ions of the protonated molecule of enniatin B (m/z 640).

TABLE 1. Source of *Fusarium tricinctum* strains tested for enniatin production

Strain (NRRL no.)	Culture collection (no. or source of supplier)	Geographic location	Habitat	Disease symptom	Enniatin estimation	
					TLC[a]	MS/MS (μg/g)
26430	R. T. Kane (12)	New York	Winter wheat	Crown and root rot	3	3,270
26562	R. T. Kane	New York	Winter wheat	Crown and root rot	3	490
26563	R. T. Kane	New York	Winter wheat	Crown and root rot	2	20
26564	R. T. Kane	New York	Winter wheat	Crown and root rot	3	1,630
26565	R. T. Kane	New York	Winter wheat	Crown and root rot	1	ND
26506	ATCC 38179 (11)	Florida	English ivy	Leaf spot	3	700
26507	ATCC 38180	Florida	English ivy	Leaf spot	3	100
26508	ATCC 38181	Florida	English ivy	Leaf spot	ND	ND
26509	ATCC 38182	Florida	English ivy	Leaf spot	3	84
26510	ATCC 38183	Florida	English ivy	Leaf spot	1	47
13434	T-387[b]	Germany	Red clover	···	1	3
13435	T-388	Finland	Barley	···	ND	ND
13442	T-546	Australia	Pasture soil	···	3	1,540

[a] Thin-layer chromatographic evaluation of enniatin production: ND = not detectable; 1 = barely detectable; 2 = intermediate; 3 = easily detectable.
[b] *Fusarium* Research Culture Collection, The Pennsylvania State University, College Park.

TABLE 2. Evaluation of the effect of enniatin on leaf and root development of germinating wheat seeds

Treatment	Six-day growth (cm)				
	Leaf		Root		
	Length[a,b]	Control (%)	Length[a,b]	Control (%)	Ratio L/R
Water control	8.7	···	11.8	···	0.74
1% DMSO control	8.1	100	10.7	100	0.76
5 μg/ml enniatin	8.6	106	10.2	95	0.84
10 μg/ml enniatin	8.3	102	9.8	92	0.85
20 μg/ml enniatin	7.8	96	7.9	74	0.98
40 μg/ml enniatin	6.7	82	5.7	53	1.18
80 μg/ml enniatin	5.1	63	3.8	36	1.34

[a] Average of 41–48 germinating seeds.
[b] Increased levels of enniatin were associated with decreased levels of both leaf and root development. Linear regression equations were:

Leaf length = 8.623 cm − 0.045 cm/μg/ml, $R^2 = 0.46$
Root length = 10.725 cm − 0.097 cm/μg/ml, $R^2 = 0.76$

(Regression coefficients were nonzero, $P < 0.001$).

carbon signals appeared as the molecular weight suggested. A complex doublet at 5.125 ppm (1 H) was observed for the methine proton between the ester oxygen and the amide carbonyl. The methine proton between the amide nitrogen and the ester carbonyl of enniatin B was seen as a complex signal at 4.527 ppm (1 H). A smaller signal at 4.71 (about 0.2 H) can be attributed to the same methine on enniatins at *m/z* 654, which contain one longer homolog and two *N*-methyl isoleucines. The *N*-methyl protons are seen as a broad singlet at 3.11 ppm (3 H). Two broad singlets at 2.255 (1 H) and 2.022 (1 H) are from the methines on the isopropyl groups, and a complex multiplet centered around 0.956 ppm (12 H) arises from the nonequivalent methyl groups on the isopropyl groups.

Quantitation of production by strains. MS/MS semiquantitative analyses revealed a high level (>1.0 g/kg) of enniatin from three strains of *F. tricinctum*, NRRL 26430, NRRL 26564, and NRRL 13442, and a lower level (<700 mg/kg from seven of the 10 remaining strains [Table 1]).

From NRRL 26430, enniatin B and trace amounts of an enniatin at *m/z* 654 were recovered together as white crystals in amounts of about 1–3 g/kg of substrate. The crystals from acetonitrile had an R_f of about 0.5 when developed in chloroform:methanol and about 0.7 in ethyl acetate:methanol:water. Crystals melted at 166 or 167 C and were soluble in methanol, ethanol, acetone, and boiling acetonitrile, but they were not soluble, or only sparingly soluble, in hexane and acetonitrile at room temperature. The minimum amount detectable with iodine vapor was 250 ng. This level of enniatin was barely visible as a yellow spot, whereas 125 ng could not be seen.

Effect on wheat seed germination. A phytotoxic effect of enniatin was evidenced by reduced growth of germinating wheat seeds. Increased levels of enniatin were associated with decreased levels of both leaf and root development (Table 2). Root elongation was retarded to a greater extent (slope = 0.097 cm/μg/ml) than leaf elongation (slope = 0.045 cm/μg/ml).

DISCUSSION

Several *F. tricinctum* strains produced relatively large amounts of enniatin B and trace amounts of an enniatin at *m/z* 654 (Fig. 2A). Although this is the first report of enniatin production by a species in the *Fusarium* section Sporotrichiella, the diversity of substrates and geographic locations suggest that enniatin biosynthesis is at least as common in *F. tricinctum* as in other producing *Fusarium* species. Enniatins are known metabolites of species representing five of the 12 *Fusarium* sections sensu Marasas et al (14), and there is an unverified report that *F. moniliforme* Sheldon (Liseola) may also make enniatin (1). Because only a limited number of surveys employing TLC and detection of enniatin with iodine vapor have been conducted, it is questionable that these metabolites can still be considered restricted in distribution as suggested by others (3,19). It is highly probable that additional strains and species not yet reported as enniatin producers would be found to possess these phytotoxic substances if more surveys were conducted in which diverse growth conditions and sensitive analytical procedures were used. In our limited survey of 13 *F. tricinctum* strains, 10 strains were found to produce enniatin by MS/MS analyses, and 11 strains were positive by TLC. Because MS/MS is about 200 times more sensitive than TLC (Table 1), anomalies are most likely due to misidentifying substances with R_f values near to those of enniatin.

The present study was initiated to evaluate *F. tricinctum* sensu Marasas et al (14) for toxin production, but once the phytotoxic metabolite enniatin was identified from isolates of winter wheat with root and crown rot (12), an in vitro test to determine if it could be a factor in plant development was added. Root and crown rot of wheat is a complex disease usually involving more than one pathogen, with *Bipolaris sorokiniana* (Sacc. in Sorok) Shoem., *F. graminearum* Schwabe, and *F. avenaceum* being primary pathogens and *F. tricinctum* a secondary invader (12). Interestingly, the species of *Fusarium* associated with root and crown rot contain strains that are potential producers of enniatin and/or trichothecenes (22). Before the discovery that enniatin is a metabolite of *F. tricinctum*, the 13 strains studied were analyzed for trichothecenes after growth on solid and liquid media; none was found. The lack of trichothecenes in these cultures is in

agreement with Marasas et al (14), who report that of the *F. tricinctum* studied, none produced any known mycotoxin. In addition, Vesonder and Hesseltine (22) do not list any phytotoxic metabolite other than trichothecenes from the four species in the section Sporotrichiella. Of the species in the section Sporotrichiella, the trichothecenes are only biosynthesized by *F. sporotrichioides* and *F. poae*; therefore, in the restricted classification of Marasas et al (14), enniatins are probably the only known toxins elaborated by *F. tricinctum*. In 1960, Gäumann et al (8) demonstrated that enniatin has deleterious effects on the water maintenance of excised tomato shoots. Symptoms of toxicity were wilting, necrosis of leaves, and loss of turgor. Apparently, this was the only attempt to associate enniatin with disease symptoms. Our germinating wheat seeds did not show any wilt symptoms.

In the case of enniatin, a phytotoxin produced by strains in many pathogenic species of fusaria, knowledge gained concerning its activity in a disease could likely be used to explain its role in other plant diseases where the invading fungus is a different species but produces the same metabolite. The presence of enniatin in *F. tricinctum* strains isolated from a plant disease with a definable symptom such as leaf spot on English ivy is a possible model in defining the role of enniatin, if any, in plant diseases without conspicuous symptoms such as stunting. Analysis of fungal strains for the presence of phytotoxins by MS/MS allows a selection, with confidence, of producing and nonproducing strains.

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